SUMOylation of DISC1:

a potential role in neural progenitor proliferation in the developing cortex

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Abstract

DISC1 is a multifunctional, intracellular scaffold protein. At the cellular level, DISC1 plays a pivotal role in neural progenitor proliferation, migration, and synaptic maturation. Perturbation of the biological pathways involving DISC1 is known to lead to behavioral changes in rodents, which supports a clinical report of a Scottish pedigree in which the majority of family members with disruption of the *DISC1* gene manifest depression, schizophrenia, and related mental conditions. The discrepancy of modest evidence in genetics but strong biological support for the role of DISC1 in mental conditions suggests a working hypothesis that regulation of DISC1 at the protein level, such as posttranslational modification, may play a role in the pathology of mental conditions. In this study, we report the SUMOylation of DISC1. This posttranslational modification occurs on lysine residues where small ubiquitin-related modifier (SUMO) and its homologs are conjugated to a large number of cellular proteins, which in turn regulates their subcellular distribution and protein stability. By using *in silico*, biochemical, and cell biological approaches, we now demonstrate that human DISC1 is SUMOylated at one specific lysine 643 (K643). We also show that this residue is crucial for proper neural progenitor proliferation in the developing cortex.

Introduction

Over the past several decades, we have used genetic and neuropathological approaches to explore compelling molecular leads to address the mechanisms of neurological and neuropsychiatric disorders [1-3]. For example, tau and α-synuclein proteins have been underscored as major components of inclusion bodies that occur in the brains of patients with Alzheimer's disease and Parkinson's disease, respectively [4, 5]. The tau and α-synuclein proteins are pathologically phosphorylated [6, 7], and the pathological roles of these posttranslational modifications have been elucidated [8]. Likewise, pathological implications of other posttranslational modifications have also been reported [9].

The *DISC1* locus has received broad attention in the context of psychiatric illness, since a balanced translocation that segregated in a Scottish pedigree with schizophrenia and depression was reported [10]. Since the discovery of the gene, many neurobiologists have studied the biological function of DISC1. At the cellular level, DISC1 plays a pivotal role in neural progenitor proliferation, migration, and synaptic maturation [11, 12]. Perturbation of the biological pathways involving DISC1 is known to lead to behavioral changes in rodents [13-21]. In contrast to biological results that support the role for DISC1 in mental conditions, genetic evidence to support the contribution of the *DISC1* gene in sporadic schizophrenia has been modest [22]. The discrepancy of modest evidence in genetics but strong biological support for the role of DISC1 in mental conditions led us to propose a working hypothesis that regulation of DISC1 at the protein level, such as posttranslational modification, may play a role in its function in pathology.

DISC1 is located in multiple subcellular domains, including the centrosome, postsynaptic density, and the nucleus [11, 23, 24]. Like tau and α-synuclein, specific phosphorylation on DISC1 affects its localization to the centrosome and plays a crucial role in neurodevelopment [25]. Protein targeting to the nucleus is also influenced by posttranslational modifications, such as SUMOylation [26]. SUMOylation occurs on lysine residues whereby small ubiquitin-related modifiers (SUMO) and homologs are conjugated to proteins [27]. SUMOylation has emerged as a major regulator of nuclear

function, including DNA replication, DNA damage response, and transcription [28-30]. Furthermore, an important role for SUMOylation in protein trafficking to the nucleus has also been reported [31].

In the present study, we show that human DISC1 is SUMOylated at lysine 643 (K643) with *in silico*, biochemical, and cell biological approaches. This specific SUMOylation site of DISC1 regulates neural progenitor proliferation in the developing cortex.

Methods

Animals

Pregnant C57BL/6 mice were purchased from Charles River.

Constructs and antibodies

A K643A variant of human DISC1 or a K640A variant of mouse DISC1 (mDISC1) was introduced by PCR-based mutagenesis as mentioned previously [25]. All constructs in the present study were created as described previously [24, 32]. We used short hairpin RNA (shRNA) construct of DISC1 that was published previously (targeting sequence is 5'-GGCAAACACTGTGAAGTGC-3') [24]. We used a control RNAi construct with a scrambled sequence without homology to any known messenger RNA [33]. GFP-tagged SUMO1, FLAG-tagged SUMO1, and Myc-tagged Ubc9 constructs were generous gifts from Dr. Pandolfi (Beth Israel Deaconess Cancer Center, USA), Dr. Hayward (Johns Hopkins University, USA), and Dr. Matunis (Johns Hopkins School of Public Health, USA), respectively. The following antibodies were used: mouse monoclonal anti-HA antibody (Covance); rabbit polyclonal anti-FLAG antibody (Sigma); rabbit polyclonal anti-SUMO1 antibody (Zymed); mouse monoclonal anti-BrdU antibody (BD Biosciences); anti-DISC1-C2 antibody [34]; and a rabbit polyclonal anti-human DISC1 antibody (kindly provided by Dr. Akiyama, University of Tokyo) [35-37].

In silico prediction of SUMOylation site(s) in DISC1

Prediction of DISC1 SUMOylation sites was determined by using the SUMOplot™ Analysis Program from ABGENT available at http://www.abgent.com/sumoplot. The score indicates the probability that the targeted lysine is SUMOylated.

Recombinant proteins

To obtain C-terminal DISC1 fragmented glutathione S-transferase (GST) fusion proteins, the C-terminal of DISC1 (amino acids 598-854) was inserted into pGEX vector. The expression plasmids were introduced into BL21 competent E. coli with 0.1 mM IPTG. Recombinant proteins were purified from E. coli with glutathione sepharose or amylase beads.

In vitro SUMOylation assay

In vitro SUMOylation was assayed by use of the SUMOylation assay kit (Boston Biochem). Briefly, reactions contained 10 μg of GST-DISC1-C, 50 μM SUMO proteins, 100 nM SUMO activating enzyme, E1, 5 μM Ubc9, and 1mM Mg-ATP in buffer (50 mM HEPES, pH 8.0, 100 mM NaCl, 1 mM DTT), and were incubated at 37˚C for 2 h. The reactions were also run in the absence of ATP as a negative control. Proteins were separated on a NuPAGE 4-12% Bis-Tris gel (Invitrogen) and visualized with SimplyBlue SafeStain (Invitrogen). For Western blotting, an 8% Tris-Glycine gel (Invitrogen) was used.

Ubc9 fusion-directed SUMOylatin (UFDS) assay

UFDS assay was performed as previously described [32] with minor modifications. Briefly, HEK293 cells were transfected with the construct of DISC1-Ubc9 or DISC1-Ubc9 (C93S) together with the SUMO1-GFP construct, and lysed 48 h after transfection. Immunoblots were conducted with the anti-DISC1 antibody described above [35].

Cell culture

COS7 cells were used to assay SUMOylation on DISC1. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS. The expression constructs were transfected into cells by using FuGENE®6 (Roche Applied Sciences). U373 cells were transfected with a DISC1 or DISC1-K643A construct to examine the nuclear translocation of SUMOylated DISC1. Cells were immunostained as previously described [25]. The intensity of immunostaining of DISC1 in the nucleus versus the whole cell was quantified with Metamorph (Molecular Devices) for all experimental groups. The intensity ratio of the signal for at least 30 cells per group was analyzed in three independent experiments.

Immunoprecipitation

Cells were lysed in a RIPA buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT, 1 mM PMSF, 1 mM EDTA, 1% Triton X-100) containing protease inhibitor mixture (Roche Applied Sciences). Supernatant fractions were obtained from lysates after centrifugation at 13 000 rpm for 15 min. Soluble fractions were used for immunoprecipitation as described previously [25].

In utero electroporation

Pregnant C57BL/6 mice at embryonic (E) day 13 were deeply anesthetized by intraperitoneal administration of 2,2,2-tribromoethanol in tert-amyl alcohol (0.4 mg/g), and intrauterine embryos were surgically manipulated as described previously [24, 38]. Plasmid solutions in volumes of 1-2 μl containing RNAi plasmids (2 μg/μl) together with CAG-driven mDISC1 with mutations in RNAi targeting site expression vector (1 μg/μl) and/or CAG-driven GFP expression vector (1 μg/μl) were injected into the lateral ventricles. To use the same amount of DNA for each condition, an empty expression vector (pCAGGS1) was used as necessary. Electronic pulses (35 V, 50 ms, 4 times) were applied using an electroporator (CUY21E, Tokiwa Science) with forceps-type electrode (CUY650-5, Tokiwa Science).

BrdU incorporation assay and brain slice preparation

Incorporation of BrdU was assayed was performed as previously described [25] with minor modifications. 50 mg/kg BrdU (Sigma) was injected intraperitoneally into E13 pregnant mice 48 h after electroporation. Then 2 h after BrdU injection, brains were fixed with 4% paraformaldehyde, and 20 μm coronal sections were obtained with a cryostat (CM 1850, Leica). For BrdU immunostaining, brain slices were incubated in 2 N HCl at 37˚C for 30 min before incubation with mouse anti-BrdU antibody. Images of the slices were acquired with a confocal microscope (Zeiss LSM510 and Olympus FV300).

Statistical analysis

To compare four groups, one-way ANOVA followed by Bonferroni post hoc for multiple comparisons test was used. $p < 0.05$ were considered to be statistically significant ($*p < 0.05$).

Results

SUMOylation of C-terminal DISC1 at lysine residue 643 (K643) in vitro

DISC1 is found at multiple subcellular domains, and its nuclear localization and biological roles have been demonstrated [22]. This subcellular targeting may be determined in part by posttranslational modifications [25]. As a role of SUMOylation for targeting proteins to the nucleus is known [26], we hypothesized that a pool of DISC1 may be SUMOylated. We thus first predicted the SUMOylation site(s) in DISC1 by using a bioinformatics approach with the ABGENT SUMOplot (http://www.abgent.com/sumoplot). By this method, DISC1 at lysine residue 643 (K643) was identified as a strong candidate SUMOylation site (**fig. 1a**). Since K643 is in the C-terminus of DISC1, we assayed *in vitro* SUMOylation with GST-tagged C-terminal DISC1 protein spanning amino acid residues 598-854 (DISC1-C). In the presence of ATP, which is crucial for SUMO-protein conjugation, DISC1-C was SUMOylated by SUMO1, SUMO2, or SUMO3 (**fig. 1b**). Next, to confirm whether K643 is essential for

SUMOylation in DISC1-C, we performed *in vitro* SUMOylation assays using mutant DISC1-C where lysine residue 643 was substituted into alanine (DISC1-C-K643A). Even in the presence of ATP, DISC1-C-K643A was not SUMOylated by SUMO1 (**fig 1c**). These results were consistent after normalization of the SUMOylated DISC1-C signals by the amount of non-SUMOylated form of DISC1-C (cyan arrow head in **fig. 1c**). Thus, these results indicate that C-terminal DISC1 protein is SUMOylated at lysine residue 643 *in vitro*.

SUMOylation of DISC1 at K643 in cells

Next, to test whether the SUMOylation of DISC1 occurs in cells, we exogenously expressed DISC1-C together with SUMO1 in cells and examined whether DISC1-C is conjugated to SUMO. DISC1-C and SUMO1 were indeed co-immunoprecipitated, and a signal for the immunoprecipitated protein complex was observed (red arrowhead in IP panel in **fig. 2a**). Of note, a signal for SUMOylation of DISC1-C was also observed in the lysates (red arrowhead in Input panel in **fig. 2a**). Then, to determine whether K643 is the SUMOylation site in DISC1-C in cells, we examined whether the mutation in which lysine residue 643 was replaces with alanine (K643A) in C-terminal of DISC1 interfered with the SUMOylation. Immunoblotting with cells transfected with DISC1-C showed a strong signal that represents SUMOylated DISC1-C, whereas such a signal was abolished in cells expressed with DISC1-C-K643A (**fig. 2b**). These results suggest that the SUMOylation of DISC1-C occurs at lysine residue 643 in cells.

We next assessed whether the full-length DISC1 could also be SUMOylated in cells. Strong signal that represents SUMOylated DISC1 was detected when DISC1-Ubc9, which enhances the SUMOylation, was exogenously expressed together with SUMO1 in cells (red arrowhead). This SUMOylation on DISC1 was abolished by co-expression of SUMO1 with DISC1-Ubc9 (C93S), a dominant negative form of Ubc9 (**fig. 2c**).

To address the question whether K643 is the main SUMOylation site on full-length DISC1, we compared potential SUMOylation between wild-type full-length DISC1 (DISC1) and full-length DISC1-

K643A (DISC1-K643A). Cells were co-expressed with DISC1 or DISC1-K643A together with SUMO1 and Ubc9. DISC1 and SUMO1 were co-immunoprecipitated, and a signal for the immunoprecipitated protein complex was observed (red arrowhead in IP panel in **fig. 2d**), whereas such a signal was abolished in cells expressed with DISC1-K643A. These results were consistent after normalization of the SUMOylated DISC1 signals by the amount of non-SUMOylated form of full-length DISC1 (cyan arrow heads in **fig. 2d**). Of note, a signal for SUMOylation of DISC1 was also observed in the lysates (red arrowhead in Input panel in **fig. 2d**). On the basis of these results, K643 is considered the main SUMOylation site on full-length DISC1.

K640 SUMOylation site in mouse DISC1 (mDISC1) (corresponding to K643 in human DISC1) is crucial for proper proliferation of neural progenitor cells (NPCs) in the developing cortex

As DISC1 is reportedly located in multiple subcellular domains [11, 23, 24], we tested possible influence of SUMOylation on the subcellular distribution of DISC1. We observed decreased nuclear targeting of DISC1-K643A compared to wild-type DISC1, suggesting that SUMOylation at K643 in human DISC1 (corresponding to K640 in mouse DISC1) may play a role in the nuclear localization of DISC1 (online **suppl. fig. 1**).

Previous studies have shown that DISC1 modulates NPCs proliferation *in vivo* [25, 39]. Thus, We tested how the K640 SUMOylation site in mDISC1 affected the proliferation of NPCs by *in utero* gene transfer combined with BrdU pulse-labeling. Consistent with our previous study [25], the proportion of cells incorporating BrdU was significantly decreased in brains with mDISC1 knockdown (mDISC1 RNAi) compared to control, indicating that NPC proliferation was impaired. These knockdown effects were ameliorated by co-expression with wild type mDISC1, but not by mDISC1-K640A (**fig. 3**), suggesting that the K640 SUMOylation site in mDISC1 is crucial for proper proliferation of NPCs in the developing cortex.

Discussion

Here we report that DISC1 protein is SUMOylated. By combining *in silico*, biochemical, and cell biological approaches, we have determined that lysine 643 in human DISC1 (which corresponds to lysine 640 in mouse DISC1) is a major SUMOylation site. We show that mutation in this residue leads to cellular deficits, including improper neural progenitor proliferation in the developing cortex.

In our biochemical analysis, the levels of SUMOylation at DISC1-K643 were relatively modest. It is known that, in general, a small pool of SUMOylated protein can have significant biological effects [40]. Therefore, the small population of SUMOylated DISC1 might have important biological effects. In cell cultures, we observed that the mutation at the lysine residue for the critical SUMOylation affected nuclear localization of DISC1. Thus far, several papers have indicated a role for nuclear DISC1 [25, 39]. By examining autopsied brains from patients with neuropsychiatric conditions, Sawamura et al. [41] suggested that nuclear DISC1 in the orbitofrontal cortex may be involved in substance abuse. The Furukubo-Tokunaga lab [42] reported that DISC1 is involved in the regulation of sleep homeostasis, in particular in association with nuclear DISC1. More recently, the Tsai lab [43] has demonstrated that nuclear DISC1 is crucial for the regulation of phosphodiesterase-cyclic AMP signaling that underlies multiple psychiatric conditions. Taken together, although each study is still independent without a cohesive or integrative story of nuclear DISC1 function yet, studying nuclear DISC1 will be important in studying the pathological mechanisms for mental illnesses. We hope that SUMOylation at K643 in human DISC1 may be a solid molecular probe to lead such mechanistic studies.

In addition, we observed that the replacement of lysine with alanine at a critical SUMOylation site (mouse K640 that corresponds to human K643) disturbed neural progenitor proliferation in the developing cortex. In the experiments, we used *in utero* electroporation with DISC1-RNAi and expression constructs. The knockdown effect by the DISC1-RNAi construct used in the current study has been fully validated by multiple groups [11, 24, 25, 39]. Furthermore, DISC1 knockdown-mediated aberrant neural progenitor proliferation has been shown with multiple RNAi constructs targeting

different regions of DISC1, and been rescued by co-expressing the full-length isoform of DISC1 in the present and previous studies [25, 39]. Although the significant influence of mouse DISC1-K640 is not a direct demonstration that SUMOylation of DISC1 is crucial for the neurodevelopmental process, we believe this is very suggestive evidence. To address this question in a more direct manner, although it is beyond the scope of the present study, we may need to develop anti-SUMOylation specific DISC1 antibodies. Equivalent antibodies have been frequently used as tools to study protein phosphorylation [44], and indeed applied to a study in DISC1 in the past [25]. There is thus a successful precedence for using anti-SUMOylation specific antibodies in biomedical studies [7, 45]. Such posttranslational modification-specific antibodies may become useful probes to study the pathology of mental illness.

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Statement of Ethics

All animal care and use was in accordance with guidelines for the care and use of laboratory animals issued by the National Institutes of Health and Johns Hopkins University. The authors have no ethical conflicts to disclose.

Disclosure Statement

Nicholas Brandon is a full-time employee and a shareholder at AstraZeneca. The other authors have no conflicts of interest to report.

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Figure legends

Fig. 1. SUMOylation of C-terminal DISC1 at lysine residue 643 (K643) *in vitro*

a, DISC1 SUMOylation sites predicted by use of the ABGENT SUMOplot^{TM}.

b, *In vitro* SUMOylation assay with GST-tagged C-terminal DISC1 (DISC1-C). Proteins were separated on a 4-12 % Bis-Tris gradient gel. Coomassie blue-staining showed that DISC1-C was covalently conjugated to SUMO1, SUMO2, and SUMO3. Red arrowheads indicate SUMOylated forms of DISC1-C.

c, *In vitro* SUMOylation assay with GST-tagged C-terminal DISC1 (DISC1-C) or mutant C terminal DISC1 where K643 was substituted by alanine (DISC1-C-K643A). 8 % Tris-Glycine gel electrophoresis and immunoblotting showed that DISC1-C but not DISC1-C-K643A was SUMOylated. Red arrowheads indicate SUMOylated forms of DISC1-C.

Fig. 2. SUMOylation of DISC1 at lysine residue 643 (K643) *in cells*

a, Conjugation of HA-DISC1-C with FLAG-SUMO1 (SUMOylation of DISC1) was shown in COS7 cells. COS7 cells were transfected with HA-DISC1-C and/or FLAG-SUMO1. Cell lysates were immunoprecipitated with anti-FLAG antibody and the immunoprecipitates were blotted with anti-HA antibody. Red arrowheads indicate SUMOylated forms of DISC1-C.

b, COS7 cells were transfected with HA-DISC1-C or HA-DISC1-C-K643A. Cells with HA-DISC1-C showed a strong signal, which represents a SUMOylated form of DISC1-C, suggesting SUMOylation of DISC1 with endogenous SUMO protein. This signal was significantly abolished in cells transfected with HA-DISC1-C-K643A. Red arrowhead indicates SUMOylated forms of DISC1-C.

c, SUMOylation of full-length DISC1 was shown in HEK293 cells by Ubc9 fusion-directed SUMOylation (UFDS) assay. SUMO1-GFP fusion was co-transfected with DISC1-Ubc9, an E2 SUMO conjugating enzyme fusion, or DISC1-Ubc9 (C93S), a dominant negative form of Ubc9 fusion, in

HEK293 cells. Cell lysates were immunoblotted with anti-DISC1 antibody. Red arrowhead indicates SUMOylated forms of DISC1.

d, COS7 cells were co-transfected with HA-DISC1 or HA-DISC1-K643A together with FLAG-SUMO1 and myc-Ubc9. Cell lysates were immunoprecipitated with anti-HA antibody and the immunoprecipitates were blotted with anti-FLAG antibody. Signals for SUMOylated full-length DISC1 were observed in cells transfected with HA-DISC1 (red arrowheads), whereas almost no signals were found at the same size in cells transfected with HA-DISC1-K643A.

Fig. 3. K640 SUMOylation site in mouse DISC1 (mDISC1), corresponding to K643 in human DISC1, is crucial for proper proliferation of neural progenitor cells (NPCs) *in vivo*

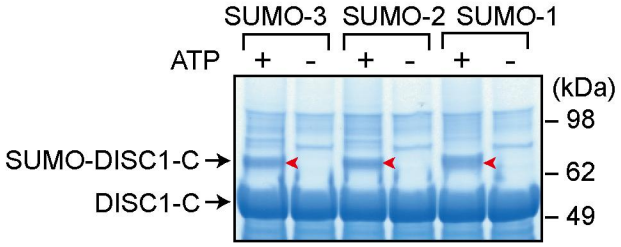
Images show E15 brains that were electroporated with GFP together with control shRNA, mDISC1 shRNA, mDISC1 shRNA + wild-type (wt) mDISC1, or mDISC1 shRNA + mDISC1-K640A at E13. BrdU was injected 2 h before brain extraction. The proportion of BrdU incorporating cells in the ventricular (VZ) and multipolar cell accumulation zone (MAZ) [46] in brains with mDISC1 shRNA, was significantly lower than that in brains with control shRNA. This phenotype was rescued by wt mDISC1 but not by mDISC1-K640A. Graph shows the proportion of GFP/BrdU double-positive cells over total GFP positive cells in the VZ/MAZ. Scale bar, 20μm. Error bars indicate s.e.m. **P*<0.05.

Fig. 1

a

No.	Pos.	Group	Score
1	K643	KKLGS~**VKED**~YNRLR	0.93
2	K372	ENDDY~**DKAE**~TLQQR	0.50
3	K768	CAGGE~**QKEE**~SYILS	0.50
4	K781	SAELG~**EKCE**~DIGKK	0.50
5	K332	WDTLL~**RKWE**~PVLRD	0.44
6	K743	HSEDK~**RKTP**~LKVLE	0.34
7	K638	SSRNV~**KKLG**~SVKED	0.31

b



c

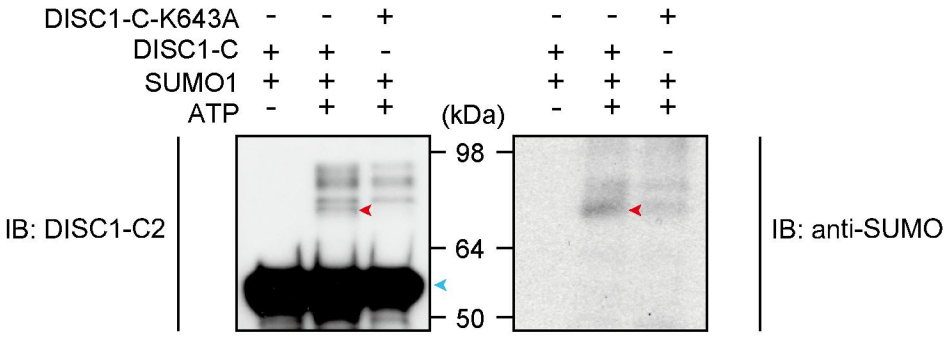
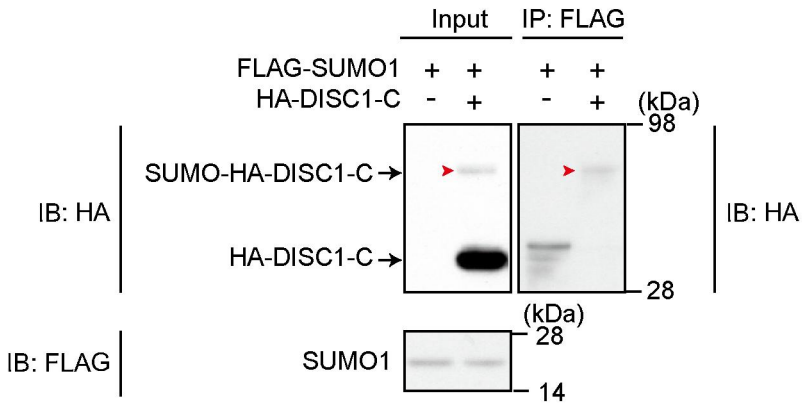
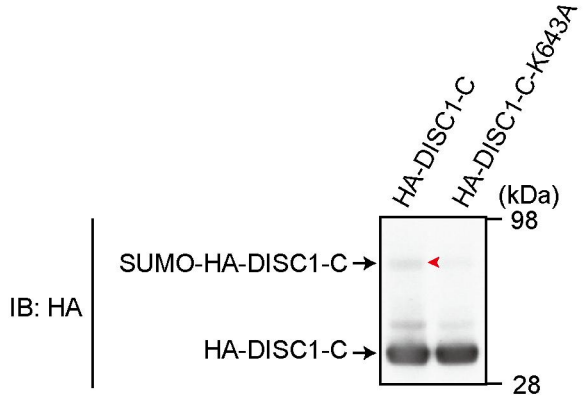


Fig. 2

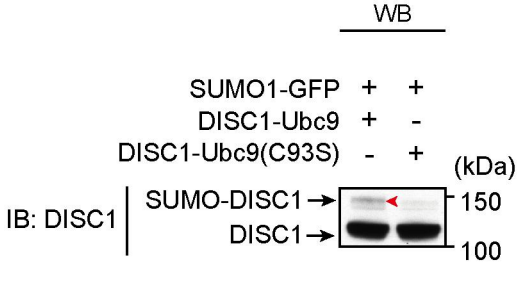
a


Input
IP: FLAG
FLAG-SUMO1 + + + +
HA-DISC1-C - + - +
(kDa)
98
28
SUMO-HA-DISC1-C
HA-DISC1-C
IB: HA
IB: HA
(kDa)
28
14
IB: FLAG
SUMO1

b


HA-DISC1-C
HA-DISC1-C-K643A
(kDa)
98
28
SUMO-HA-DISC1-C
HA-DISC1-C
IB: HA

c


WB
SUMO1-GFP + +
DISC1-Ubc9 + -
DISC1-Ubc9(C93S) - +
(kDa)
150
100
IB: DISC1
SUMO-DISC1
DISC1

d

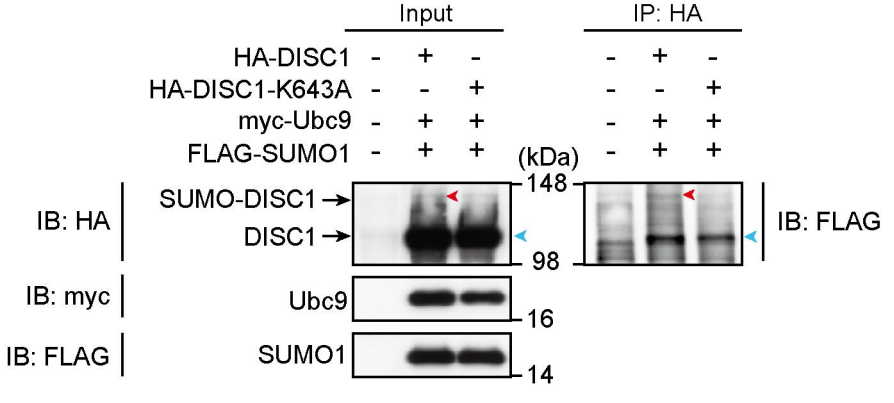
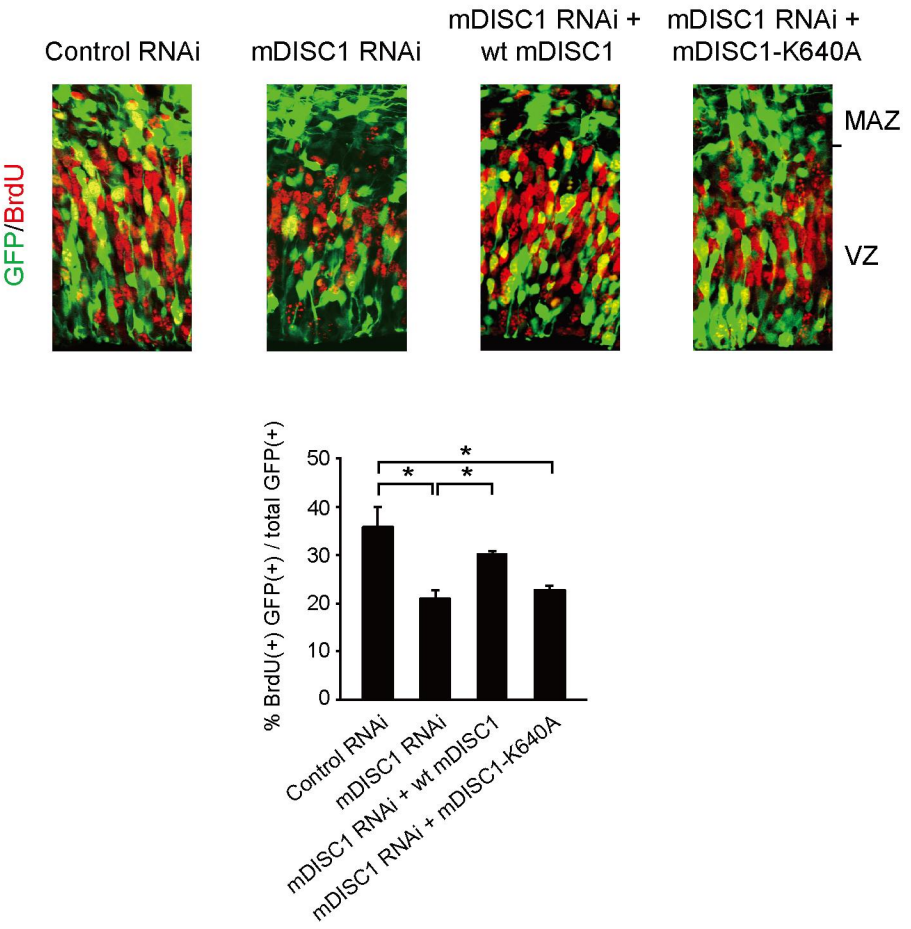
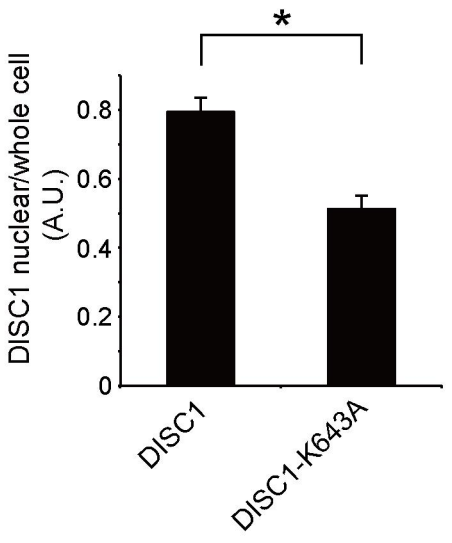

Input
IP: HA
HA-DISC1 - + -
HA-DISC1-K643A - - +
myc-Ubc9 - + +
FLAG-SUMO1 - + +
(kDa)
148
98
16
14
IB: HA
SUMO-DISC1
DISC1
IB: myc
Ubc9
IB: FLAG
SUMO1
IB: FLAG

Fig. 3


Control RNAi
mDISC1 RNAi
mDISC1 RNAi + wt mDISC1
mDISC1 RNAi + mDISC1-K640A
GFP/BrdU
MAZ
VZ
% BrdU(+) GFP(+) / total GFP(+)
50
40
30
20
10
0
*
*
*
Control RNAi
mDISC1 RNAi
mDISC1 RNAi + wt mDISC1
mDISC1 RNAi + mDISC1-K640A



Suppl. Fig.1. K643 SUMOylation site in human DISC1 is crucial for nuclear translocation Graph represents the immunoreactivity of DISC1 inside the nucleus. DISC1-K643A showed significantly decreased nuclear localization compared to wild-type DISC1. Error bars indicate s.e.m. *P<0.05.